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Lassa and Mopeia Virus Replication in Human Monocytes/Macrophages and in Endothelial Cells: Different Effects on IL-8 and TNF-α Gene Expression

Igor S. Lukashevich,[1] Raisa Maryankova,[2] Alexander S. Vladyko,[2] Natalia Nashkevich,[3] Svetlana Koleda,[3] Mahmoud Djavani,[1] Douglas Horejsh,[1] Nikolai N. Voitenok,[4] and Maria S. Salvato[1]*

[1]*Department of Pathology, University of Wisconsin, Madison, Wisconsin*
[2]*Belarussian Research Institute of Epidemiology and Microbiology, Minsk, Belarus*
[3]*Institute of Hematology and Blood Transfusion, Minsk, Belarus*
[4]*Molecular Hematology and Immunology Fund, Moscow, Russia*

Cells of the mononuclear and endothelial lineages are targets for viruses which cause hemorrhagic fevers (HF) such as the filoviruses Marburg and Ebola, and the arenaviruses Lassa and Junin. A recent model of Marburg HF pathogenesis proposes that virus directly causes endothelial cell damage and macrophage release of TNF-α which increases the permeability of endothelial monolayers [Feldmann et al., 1996]. We show that Lassa virus replicates in human monocytes/macrophages and endothelial cells without damaging them. Human endothelial cells (HUVEC) are highly susceptible to infection by both Lassa and Mopeia (a non-pathogenic Lassa-related arenavirus). Whereas monocytes must differentiate into macrophages before supporting even low level production of these viruses, the virus yields in the culture medium of infected HUVEC cells reach more than 7 log10 PFU/ml without cellular damage. In contrast to filovirus, Lassa virus replication in monocytes/macrophages fails to stimulate TNF-α gene expression and even down-regulates LPS-stimulated TNF-α mRNA synthesis. The expression of IL-8, a prototypic proinflammatory CXC chemokine, was also suppressed in Lassa virus infected monocytes/macrophages and HUVEC on both the protein and mRNA levels. This contrasts with Mopeia virus infection of HUVEC in which neither IL-8 mRNA nor protein are reduced. The cumulative down-regulation of TNF-α and IL-8 expression could explain the absence of inflammatory and effective immune responses in severe cases of Lassa HF. ***J. Med. Virol. 59:552–560, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: viral hemorrhagic fevers; Lassa virus; inflammatory cytokines; TNF-α; IL-8

INTRODUCTION

Lassa virus is a widespread human pathogen in West Africa. Infection can be asymptomatic or result in disease varying from mild influenza-like illness to a severe hemorrhagic fever (HF). Both viral and host-derived factors are involved in the pathogenesis and clinical outcome of the infection in man. Lassa virus belongs to the Old World group of the *Arenaviridae* which includes the prototypic lymphocytic choriomeningitis virus (LCMV) with moderate pathogenic potential and the Mopeia, Mobala and Ippy viruses with mild or unknown pathogenic potentials. The pathogenesis of Lassa HF is not understood, but it has been clearly shown that the well-studied LCMV murine infection is an inappropriate model for arenaviral HF in humans [Peters, 1997; Salvato and Rai, 1998].

Lassa HF has several pathological manifestations in common with other severe viral HFs. Accumulating evidence suggests that the monocyte/macrophage system and endothelial cells are involved and play an important role in pathogenesis of arenaviral HFs as well as in filovirus (Marburg, Ebola) HFs in man. Virus-induced dysfunction of endothelial cells and virus-induced macrophage TNF-α that increases permeability of vascular endothelia have been suggested recently as a pathogenic mechanism of Marburg HF [Schnittler et al., 1993; Feldmann et al., 1996]. It has been shown that a trans-membrane Ebola glycoprotein interacts di-

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*Correspondence to: Maria S. Salvato, Ph.D., Department of Pathology, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706. E-mail: msalvato@facstaff.edu

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rectly and specifically with vascular endothelial cells in vitro and is probably involved in virus-induced endothelial cell damage [Yang et al., 1998]. In man, defective humoral responses and extensive intravascular apoptosis were associated with fatal outcome in Ebola HF [Baize et al., 1999]. Disturbance of endothelial function has been observed also in human beings suffering from Lassa HF [Fisher-Hoch et al., 1987], but the mechanism remains unknown.

Lassa is a pantropic virus in humans; however, pathohistological examinations of Lassa HF patients showed the absence of a strong inflammatory response or remarkable morphological lesions which would explain functional disorders and eventual death. Very little has been learned from necropsies of fatal Lassa cases. Electron microscopy also did not show extensive cellular alterations suggesting that widespread disturbance of cellular functions was more important than direct virus-induced structural damage [Fisher-Hoch, 1993; Peters, 1997; Peters et al., 1987].

In primates infected with Lassa virus, fatal outcome was strongly correlated with high viremia, illustrating a striking parallel with human disease. In spite of the extensive virus replication in tissues, histopathological changes were rather mild. In addition, monkey studies revealed platelet function suppression, disturbance of endothelial cells, and loss of lymphocyte and neutrophil functions [McCormick et al., 1987; Peters et al., 1987; Roberts et al., 1989; Fisher-Hoch, 1993; Fisher-Hoch et al., 1987]. At least one of the affected functions of these cells seems to be inflammatory infiltration. Necropsy studies in humans and primates found no evidence of inflammatory reaction in Lassa virus infected tissues, suggesting that the function of proinflammatory cytokines and chemokines may be suppressed during Lassa virus infection.

Lassa and Mopeia viruses share a common rodent host (*Mastomys*), cross-react with polyclonal sera, share more than 75% amino acid identity and can form reassortants after coinfection of susceptible cells. However, Lassa is the cause of HF, while Mopeia is not associated with any known human or primate disease [Peters et al., 1987; Wilson et al., 1991; Lukashevich, 1992].

We report on the replication of Lassa and Mopeia viruses in human monocytes/macrophages and endothelial cells, and on the effects of viral infection on the expression of mRNA encoding the proinflammatory cyto/chemokines TNF-α and IL-8. TNF-α is mainly produced by monocytes/macrophages and also, to a lesser extent, by CD4+ and CD8+ T lymphocytes. IL-8 is a prototypic CXC chemokine which promotes inflammatory reactions by enhancing leukocyte adherence to vascular endothelium cells, extravasation of leukocytes and the migration of these cells into tissue sites of virus replication [Ben-Barush et al., 1995; Rollins, 1997]. The results associate Lassa virus infection with suppression of TNF-α and IL-8. In contast, Mopeia virus does not down-regulate IL-8 expression in infected endothelial cells.

MATERIALS AND METHODS

Cell Cultures and Viruses

Peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood of healthy human volunteers by gradient centrifugation on Ficoll-Paque (Sigma, St. Louis, MO) and were resuspended in RPMI 1640 with 5% heat-inactivated human serum (type AB, Sigma). Monocytes/macrophages were separated from nonadherent cells in 12-well culture plates or in 25-cm^2 (T25) flasks (Falcon) by incubation for 1 hour at 37°C and subsequent washing to remove nonadherent cells. To stimulate differentiation of monocytic cells into macrophage-like cells, adherent cells were incubated in RPMI 1640 containing 100 ng/ml of phorbol ester, PMA (Sigma), for 2 hours and washed before virus infection or simply cultured in RPMI for 6–7 days with 1–2 washes during cultivation. At the end of the activation or cultivation period monocytic cells had a macrophage-like morphology. The presence of mature monocyte-derived macrophages (MDM) was confirmed by indirect immunofluorescence with a monoclonal antibody to CD14 (Dako, Germany).

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (Walkerville, MD) as a cryopreserved stock No. 2991 and subcultured according to the manufacturer's instructuctions in Endothelial Cell Growth Medium, EGM (Clonetics).

Human MDM and HUVEC cultures in T25 flasks or in 12-well tissue dishes were infected with Lassa (Josiah) or with Mopeia (AN20410) viruses. Virus stocks, infection procedures and virus titration by plaque assay on Vero E6 cells have been described previously [Lukashevich, 1992]. Cells were infected with viruses at either low (0.1 – 0.01 pfu/cell) or high (5 pfu/cell) multiplicity of infection (MOI). Culture medium of Vero E6 cells was used as "a virus free stock" in all mock-infection experiments. After adsorption (1 hour at 37°C) the inoculum was removed, cells were washed twice with PBS, and MDM were cultivated in RPMI 1640 with 5% human AB serum at 37°C in a CO_2 incubator. At different time point post infection (p. i.) 0.5 ml aliquots of the culture medium from 12-well plates were collected and stored at −70°C for virus titration and ELISA.

TNF-α and IL-8 Level Determination by ELISA

TNF-α and IL-8 release in culture medium was measured by a sensitive homologous ELISA using the same monoclonal antibody for both solid and liquid phase as has been previously described [Petyovka et al., 1995]. In some experiments the Quantikine IL-8 Immunoassay (R&D Systems, Minneapolis, MN) was used. The detection limit for both ELISAs was <15 pg/ml. Natural human TNF-α and IL-8 was induced in MDM by lipopolysaccharide (LPS) from *E. coli* 055:B55 (Sigma). In the experiments with LPS induction, the infection period and subsequent incubation of MDM was performed in the presence of LPS (10 ng/ml) for 12 or 24 hours.

RNA Isolation and RT-PCR

For RNA isolation MDM and HUVEC were cultivated in T25 flasks. At different time points p. i. duplicate flasks were used for total RNA extraction using TRIZOL (GIBCO BRL) according to manufacturer's instruction. RNA was purified on RNaid Matrix (BIO 101, San Diego, CA), re-suspended in 25–50 ul of water, and quantitated by absorbance at 260 nm. The integrity of the RNA was verified by electrophoresis in a 1% agarose-formaldehyde gel. For detection of Lassa virus-specific RNA in infected cells the Access reverse transcription-polymerase chain reaction (RT-PCR) System (Promega, Madison, WI) was used as described previously [Lukashevich et al., 1997] with the Lassa GP1 primer set (5′ACCGGGG ATCCTA GGCATTT, nt 5–24, forward; 5′-ATATAATGATGACTGTTGTTC TTTGTGCA, nt 311–339, reverse). The first-strand cDNA synthesis was performed at 48°C for 45 min and cDNA was amplified for 40 cycles at 92°C for 45 sec, 52°C for 45 sec, and 72°C for 45 sec (DNA Thermal Cycler, Perkin Elmer).

Expression of TNF-α mRNA in MDM was measured using the Quantitative PCR Detection Kit (BioSource International, Camarillo, CA). Briefly, cDNA was synthesized in a 20 ul reaction containing 1 ug of total RNA , 0.5 ug of oligo (dT)12–16 nucleotide primer, 10 mM dithiothreitol, 0.5 mM dNTP, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 200 U of MMLV RT (RETROscript, Ambion, Austin, TX). After a 60 min incubation at 42°C, samples were heated at 92°C for 10 min, chilled on ice and used for PCR amplification with biotin-labeled TNF-α primers. 2,000 copies of internal calibration standard (ICS) were mixed with cDNA prior to amplification. DNA amplification was carried out in 1 × PCR buffer supplemented with 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 0.5 U of *Taq* polymerase (Promega) in a final volume of 50 ul. The reaction was amplified for 30 cycles at 95°C for 60 sec, 55°C for 60 sec, and 72°C for 60 sec. Expected product sizes for ICS and TNF-α were 432- and 382-bp, respectively. Following PCR, the TNF-α and ICS amplicons at appropriate dilution were hybridized to either ICS or TNF-α sequence-specific capture oligonucleotides in an ELISA plate, and the captured sequences were detected and quantified by addition of enzyme-streptavidin conjugate followed by substrate. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification primer set (BioSource) was used with the same RNA templates to amplify a 382-bp GAPDH amplicon which served as an endogenous control for RNA normalization in gel-based analyses.

For IL-8 mRNA quantitation relative multiplex RT-PCR with IL-8 gene-specific primers from Ambion were used (5′TTGCCAAGGAGTGCTAAAGAAC, forward and 5′GTCACTTCTACGGT CATTTG, reverse). RNA from MDM and HUVEC was reverse transcribed into cDNA as described above using random decamers and amplified with primers for 30 cycles. The QuantumRNA 18S rRNA internal standards (Ambion) were used in multiplex PCR to attenuate 18S RNA expression and to normalize RNA samples. 18S rRNA primers and 18S rRNA Competimers (Ambion) were used at a 2:8 ratio. For normalization, ^{32}P-labeled dCTP (3000 Ci/mmol) was added to PCR reactions. Following PCR and 2% agarose gel electrophoresis, specific visualized bands were cut out, dissolved in 150 ul of QX1 solution (Qiagen, Valencia, CA) for 10 min at 50°C, mixed with 5 ml of ScintiSafe cocktail (Fisher Scientific) and the incorporated radioactivity was measured in a beta-counter (Beckman).

Real-time PCR detection of IL-8 and GAPDH amplicons was done using the GeneAmp 5700 Sequence Detection System (Perkin-Elmer). The SYBR Green I PCR Core Reagent kit was used to produce fluorescent-labeled PCR products. The only alteration of the manufacturer's protocol was to raise the annealing temperature to 64°C. Human IL-8 primers were received from Ambion and GAPDH primers were synthesized as follows: 5′CCAGTGCAAAGAGCCCAAAC (forward), 5′TGACACCCATGACGAACATTC (reverse). The result of real-time PCR was expressed as the threshold cycle (C_T). The C_T represents the PCR cycle at which the reporter fluorescence raises above a set baseline threshold when the DNA amplicon is replicating exponentially. The relative level of IL-8 message was determined by comparing the IL-8 to GAPDH and standardizing all samples to cDNA from mock-infected cells. In the exponential phase, a C_T difference of 1 is a doubling in the amount of amplicon. Therefore, to determine relative message levels, two was raised to the power of ΔC_T (the difference between C_T from infected cells and C_T from uninfected cells).

RESULTS

Lassa and Mopeia viruses replicate in MDM with relatively low yields

Human MDM were infected with Lassa and Mopeia viruses at low and high MOI and culture medium was titrated by plaque assay on Vero E6 cells. As shown in Table I, the viruses did not replicate in non-stimulated monocytes from human PBMC. However, when the cells were stimulated to differentiate into macrophages either by 6–7-day incubation or by PMA treatment, they became permissive to the viruses. Both viruses replicated in MDM without visible cytopathic effects as judged by light microscopy and MTS tetrazolium dye incorporation (data not shown) and reached a relatively low level of infectious yield (< 4.5 log PFU/ml) after low and high MOI. The replication kinetics of both viruses in MDM were very similar (Table IB).

TNF-α mRNA expression is not elevated in Lassa- or Mopeia-infected MDM

MDM grown in 12 well plates were infected with virus at a high MOI, and TNF-α release in culture medium was monitored by ELISA. Neither Lassa nor Mopeia infection induced a detectable level of TNF-α release in culture medium (>15 pg/ml). In some virus-cell systems the TNF-α induction effect was detected only

TABLE I. Replication of Lassa and Mopeia Viruses Into Monocytes/Macrophages

A. Effect of the maturation of monocytes into macrophages on Lassa virus replication

Hours post infection	Virus titer in PFU/ml[a]	
	Non-matured monocytes	MDM
12	1.1×10^2	6.5×10^1
24	2.2×10^2	1.8×10^4
48	2.9×10^2	9.5×10^3
96	3.4×10^2	2.2×10^4
120	3.7×10^2	3.3×10^3

B. Kinetics of Lassa and Mopeia virus replications in MDM

Hours p. i.	Virus titer in PFU/ml[a]	
	Lassa virus	Mopeia virus
12	6.0×10^1	1.5×10^2
24	1.5×10^4	1.0×10^4
36	1.9×10^3	2.0×10^3
48	1.2×10^3	1.0×10^4
60	2.5×10^3	5.0×10^3

[a]MOI = 0.01. Titers are the average of counts from duplicate cultures.

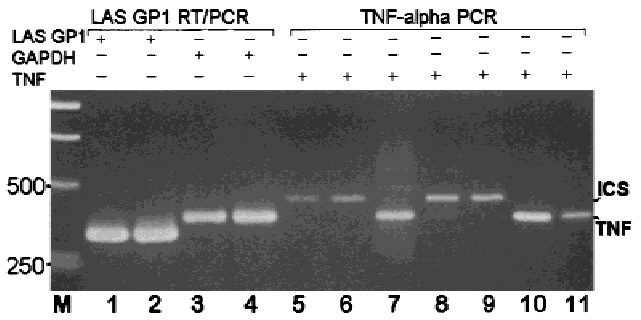


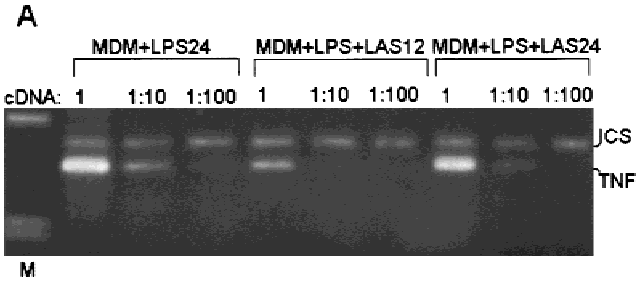
Fig. 1. RT/PCR amplification of LAS GP1 and TNF-α mRNAs in MDM. RNAs from MDM infected with LAS **(lanes 1–6)** or MOP (lanes 8-11) were extracted at 12 **(lanes 1, 3, 5, 8, 10)** or 24 **(lanes 2, 4, 6, 9, 11)** hours p. i. and used in RT-PCR with LAS GP1, GAPDH or TNF-α primers. RNA from mock-infected cells treated with LPS was used as a positive control for natural TNF-α mRNA expression (**lane 7**, 382-bp amplicon). 2,000 copies of exogenous synthesized DNA was included in PCR as TNF-α internal calibration standard, ICS (**lanes 5-8**, 482-bp amplicon). M, positions of DNA markers: ICS, TNF standard; TNF, wild type TNF-α cDNA. Note that wild type TNF-α cDNA and GAPDH cDNA amplicons have the same size, 382 bp.

at the level of TNF-α gene expression, and was not followed by protein secretion [Czarniecki, 1993]. To address this question, TNF-α expression in infected MDM was measured at the mRNA level. MDM grown in T25 flasks were infected at high MOI and TNF-α mRNA, GAPDH mRNA and Lassa GP1 mRNA were detected by RT-PCR at 12 and 24 hours p. i. As shown in Figure 1, a strong GP1-derived 340-bp band was detected in Lassa-infected MDM at 12 hours p. i. by RT-PCR indicating productive Lassa virus replication in MDM. The specificity of the PCR product was confirmed by Southern blot hybridization with the Lassa GP1-derived probe 5′-GAGGTGATGAACAT TGTTCTCATT (Accession No. JO4324) labeled with ^{32}P-dCTP (not shown).

Lassa virus infection alone did not induce PCR-detectable TNF-α mRNA expression in MDM. Under the same conditions, LPS-stimulated MDM gave TNF-α PCR products with the expected size, as determined by a TNF-α ICS. A few copies of TNF-α mRNA (less then 400 copies per ng) were detected in MDM infected with Mopeia virus at 12 hours p. i. (a faint 382 bp-band in lane 8, Fig. 1). However, this mRNA was not detectable at 24 hours p. i. It has been concluded from these experiments that Lassa infection does not increase the steady-state expression of TNF-α in human MDM in the absence of additional stimulation, from LPS for example.

Lassa Virus Infection Inhibits LPS-Induced TNF-α Expression in MDM

LPS is a strong inducer of TNF-α in human macrophages [Czarniecki, 1993; Barbara et al., 1996]. At a concentration as low as 10 ng/ml, LPS induced a high level of TNF-α mRNA expression (Fig. 1, lane 7) and released TNF-α into culture medium (30 ng/ml, not shown). TNF-α mRNA was measured by competitive PCR with TNF-α cDNA and ICS (BioSource). An example of the gel-based analysis is shown in Figure 2.



B

Amplicon	TNF-alpha mRNA copies	
	Exp. 1	Exp. 2
MDM + LPS12	23,600	13,890
MDM + LPS24	55,100	39,699
MDM + LPS + LAS12	5,436	2,353
MDM + LPS + LAS24	20,080	19,120

Fig. 2. Quantitation of TNF-α mRNA expression in human LPS-stimulated and Lassa-infected MDM by competitive PCR. **A.** Gel-based analysis of TNF-α mRNA expression in LPS-MDM infected with Lassa virus. MDM grown in T25 flasks were stimulated with LPS, infected with Lassa virus (MOI = 5) and incubated for 12 and 24 hours. At 12 and 24 hours p. i. RNA was extracted, converted into cDNA and equivalent amounts of cDNA (based on GAPDH) were amplified. Quantitative PCR was performed with biotin-labeled TNF-α primers using different concentrations of cytokine template (1, 1:10, and 1:100 dilutions) and a constant amount (2,000 copies) of ICS (BioSource). 5 ul-aliquots were analyzed on 2% agarose. M, positions of DNA markers, 500 and 250 bp. **B.** Quantitation of PCR products. 25 ul from each PCR reaction was denatured under alkaline pH and serial dilutions (1:40-1:320) of TNF-α and ICS amplicons were made in hybridization buffer in ICS- or TNF-α-specific oligonucleotide capture wells. After hybridization the captured amplicons were detected by streptavidin conjugates. Numbers of TNF-α mRNA copies were calculated as follows: Total TNF-α Optical Density (OD)/Total ICS OD × 2 × Input copy number of ICS × cDNA dilution.

The presence of TNF-α cDNA (382 bp) and ICS cDNA (482 bp) indicates proper amplification of both TNF-α and ICS templates allowing correct quantitation of TNF-α templates in the ELISA-based assay. The results of two sets of experiments are summarized in panel B (Fig. 2). LPS stimulated a greater than twofold TNF-α mRNA increase between 12 and 24 hours in

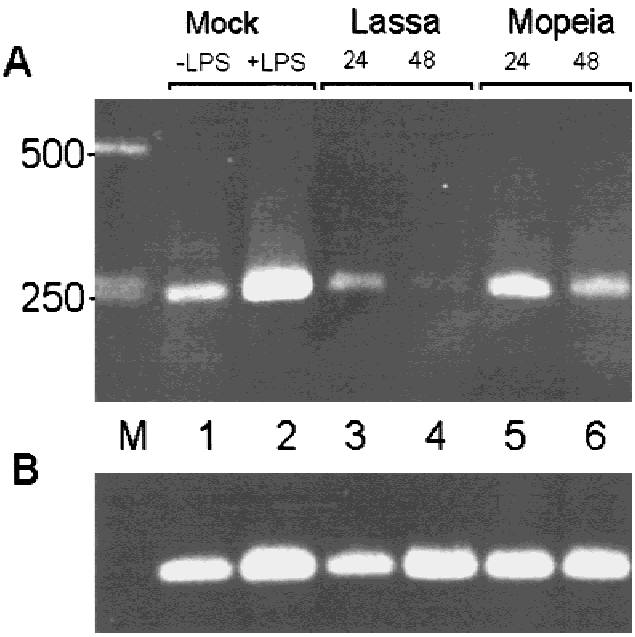


Fig. 3. IL-8 mRNA expression in human MDM infected with Lassa and Mopeia viruses. **A.** Total RNA was extracted from mock-infected cells **(lanes 1, 2)** and cells infected with Lassa **(lanes 3, 4)** and Mopeia **(lanes 5, 6)** viruses at 24 **(lanes 3, 5)** and 48 **(lanes 1, 2, 4, 6)** hours p. i. RNA was subjected by RT-PCR with IL-8 specific primers. **Lane 2**, RNA from cells stimulated with LPS, 10 ng/ml. **Lane M** denotes size markers. **B**. RT-PCR amplification of the same samples with GAPDH primers in parallel experiment.

mock-infected cells. Under the same experimental conditions, Lassa virus infection inhibited TNF-α mRNA expression in LPS-stimulated MDM. Results from duplicate experiments showed that the level of suppression varied from 77–83% at 12 hours p. i. to 52–64% at 24 hours p. i.

IL-8 Expression Is Suppressed in Virus-Infected MDM

IL-8, a prototype CXC chemokine, plays an important role in inflammation and, as it has been shown recently, in the induction of delayed-phase vascular permeability [Ben-Baruch et al., 1995; Rollins, 1997; Ward et al., 1998; Fukimoto et al., 1998]. To determine whether Lassa virus infection affects constitutive IL-8 mRNA expression, MDM were infected with the virus at a MOI of 0.1 and RNA was extracted at 24 and 48 hours p. i. The RNA converted to cDNA and equivalent amounts of cDNA (based on GAPDH) were amplified with IL-8 primers. Gel-based analyses of PCR products revealed an inhibition of IL-8 mRNA expression during Lassa and Mopeia virus replication in MDM (Fig. 3). However, the level of inhibitory effect was greater in Lassa virus infected cells.

Inhibition of IL-8 was also evident from the levels of protein expression. ELISA titration of IL-8 protein in the culture medium of infected MDM revealed the same level of inhibition for both viruses, as seen in Table II. When MDM were exposed to infectious Lassa virus (MOI = 5) and to gamma-irradiated Lassa virus

TABLE II. Levels of IL-8 in MDM Infected With Lassa an Mopeia Viruses

	IL-8, ng/ml (mean value ± SD):		
Infection	0	24	48 hours p. i.
MDM, mock	<0.015	23.36 ± 0.08	44.90 ± 0.12
MDM + LPS	<0.015	198.15 ± 4.21	217.50 ± 5.52
MDM + Lassa	<0.015	3.06 ± 0.05	1.66 ± 0.05
MDM + Mopeia	<0.015	2.47 ± 0.06	1.67 ± 0.06

(at the equivalent dose), the cells infected with inactivated virus released 2–4-fold more IL-8 than cells treated with the infectious virus suggesting that the inhibition of IL-8 release was associated with virus replication (not shown).

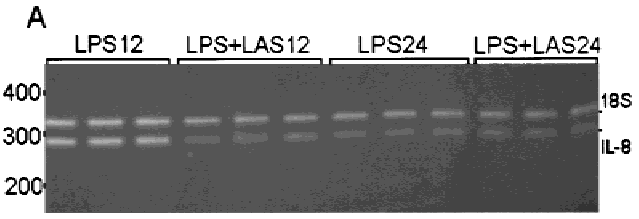
IL-8 was originally discovered as a neutrophil chemotactic factor produced by LPS-stimulated monocytes [Yoshimura et al., 1987; Schroder et al., 1987]. To investigate the effect of Lassa virus infection on LPS-induced IL-8 mRNA expression, experiments were performed with RNA extracted from LPS-stimulated MDM infected with Lassa virus at a MOI of 5. RNA was extracted at 12 and 24 hours p. i. (with or without LPS stimulation) and samples were analyzed in multiplex quantitative RT-PCR with IL-8 and 18S rRNA primers. In mock-infected LPS-stimulated cells IL-8 mRNA expression was 2-fold higher at 12 hours than at 24 hours after stimulation confirming that IL-8 induction occurred rapidly, within the first hours after stimulation with bacterial endo-and exotoxins [Krakauer, 1998]. Lassa virus infection resulted in an at least 2-fold reduction of the IL-8 mRNA expression at 12 hr after stimulated infection but did not affect the expression at the late stage of the infection of LPS-stimulated MDM (Fig. 4).

Lassa and Mopeia Viruses Effectively Replicate in HUVEC

Vascular endothelial cells are involved in the pathogenesis of some viral HFs and replication of the viruses within these cells results in either direct or indirect cellular damage with subsequent loss of vascular integrity [Peters, 1997]. Replication of Lassa and Mopeia viruses in cultured human vascular cells has not been described. To study the role of these cells in Lassa HF pathogenesis, HUVEC were grown as subconfluent monolayers and infected with Lassa and Mopeia viruses at a MOI of 0.1. As seen from Table III, the viruses infect, replicate and produce infectious progeny virions in HUVEC nearly as well as in Vero E6 cells ($1–7 \times 10^7$ pfu/ml). Both viruses replicated to high titer without cytopathic effect and the virus antigens were easily detected by IFA (not shown).

IL-8 expression Is Inhibited in HUVEC Infected With Lassa But Not With Mopeia Virus

To investigate the effect of Lassa and Mopeia infection on endothelial IL-8 synthesis HUVEC were infected with Lassa and Mopeia viruses at a high MOI. At different time points aliquots of the medium were



B

Amplicon	IL-8 CPM	IL-8/18S
MDM + LPS12	72,903+/-6,416	0.67+/-0.04
MDM + LPS+ LAS12	30,463+/-3,556	0.36+/-0.05
MDM + LPS24	29,970+/-3,677	0.33+/-0.03
MDM + LPS + LAS24	25,431+/-2,980	0.32+/-0.04

Fig. 4. Relative multiplex quantitative RT-PCR analysis of IL-8 mRNA expression in human LPS-stimulated MDM infected with Lassa virus. **A**. Gel-based analysis. cDNA from MDM RNA was synthesized and amplified with IL-8 and 18S rRNA/18S rRNA Competimers primer mix (2:8, Ambion) in replicates of three. 10 ul of each PCR was electrophoresed on a 2% agarose gel. Positions of size markers (200, 300, and 400 bp) as well as 18S rRNA cDNA and IL-8 cDNA are indicated. **B.** Semiquantitative Analysis. ^{32}P-dCTP-labeled PCR samples were visualized with ethidium bromide as shown above in panel A. IL-8 and 18S specific bands were excised from the gel and incorporated radioactivities were measured by liquid scintillation counting. To standardize IL-8 expression, radioactivity in IL-8 PCR products was divided by the corresponding 18S rRNA cDNA radioactivity in each sample. Radioactivity of the non-template control was subtracted from experimental values. Results of three independent RT-PCR experiments are summarized as radioactivity (cpm) recovered from IL-8 amplicons and as a IL-8/18S cDNA normalized values.

TABLE III. Lassa and Mopeia Virus Replication in HUVEC and Vero Cells[a]

Hours post infection	HUVEC:		VERO:	
	Lassa	Mopeia	Lassa	Mopeia
12	4.5×10^5	8.5×10^4	7.3×10^4	8.9×10^3
24	1.1×10^6	1.0×10^5	3.1×10^5	9.6×10^4
48	1.3×10^7	3.1×10^6	7.4×10^7	1.9×10^5
72	5.8×10^6	1.6×10^6	6.5×10^7	4.3×10^6

[a]MOI = 0.1. Titers are the average of counts from duplicate cultures.

collected for IL-8 ELISA. IL-8 protein accumulated gradually in culture medium of mock-infected HUVEC and reached a maximum concentration of 3.5 ng/ml at 48 hours of incubation (Table IV). In HUVEC infected with Lassa virus, IL-8 release in culture medium was suppressed over five fold by 48 hours p. i. Mopeia virus infection did not suppress IL-8 release into the culture medium of infected HUVEC. In contrast, the levels of IL-8 in Mopeia infected HUVEC were slightly higher than in mock-infected cells ($P < 0.05$ for 12, 24, and 48 hours p. i.).

IL-8 release results were confirmed at the IL-8 mRNA expression level using real-time quantitative PCR. As seen in Table V, amplification plots derived from HUVEC-Lasssa-20 hr and HUVEC-Lassa-40 hr cDNAs have different C_T values, 26.8 and 29.4, respectively. After normalization of these values to GAPDH amplicons and subtraction of HUVEC-mock C_T values, the level of the IL-8 mRNA at 40 hours was at least 2.5-fold less that at 20 hours p. i. [ΔIL-8 (40/20 hours p. i.) = $2^{1.32}$ = 2.5]. This difference was confirmed by multiplex quantitative PCR with IL-8 and 18S rRNA primers. After normalization of IL-8 radioactivity to 18S rRNAs, the IL-8/18S ratio for HUVEC-Lassa-20 hr samples was 0.51±0.09 and for HUVEC-Lassa-40 hr was 0.21±0.02.

For HUVEC-Mopeia samples after normalization and subtraction to HUVEC-mock samples the ΔIL-8 value was positive, 0.13 (Table V). This means that at 40 hours p. i. the number of IL-8 mRNA copies was 9% greater than the IL-8 copies detected at 20 hours p. i. ($0.5^{0.13}$ = 0.91).

DISCUSSION

Lassa fever virus is known to cause coagulation defects and disturbance of endothelial function [Fisher-Hoch, 1993], unlike a closely-related non-pathogenic arenavirus, Mopeia virus. We explored the possibility that these viruses differed in their ability to replicate in human monocytes/macrophages or in vascular endothelial cells, the primary cell types involved in Lassa HF pathogenesis. Replication in fresh monocytes did not occur for either virus. However, after selecting monocyte-derived macrophages (MDM) both viruses replicated equally to relatively low levels (< 4.5 $\log_{10}$ PFU/ml) without cytopathic effects on the MDM. Enhanced virus replication in MDM has also been described for Lassa virus infection of human monocytic U937 cells [Lewis et al., 1989] and for Pichinde virus infection of THP-1 cell lines [Polyak et al., 1991] suggesting that the mature cells contain a host factor essential for virus replication. Infection with HF viruses (e.g., Marburg virus) can induce endothelial cell lysis in culture. This finding has provided a simple explanation for the virus-mediated endothelial cell damage and hemorrhage observed *in vivo* [Schnittler et al., 1993]. However, direct viral lysis of endothelial cells was not observed for the arenaviruses Junin, the agent of Argentine HF [Andrews et al., 1987], or for Lassa virus [present paper] which effectively replicate in endothelial cells without cytopathicity. Lassa and Mopeia viruses are equally non-cytopathic in cultured MDM and endothelial cells. It is possible that in vivo the infection sensitizes endothelial cells to apoptotic signals from other cell types. Our data also imply that the viral infection affects function but not structure of MDM and endothelial cells.

Blood monocytes migrate into tissues where they differentiate into tissue macrophages. Due to the ability of macrophages to supply infectious virus and to migrate, these cells may serve as a source of virus for generalized infection. Also, the replication of virus in monocytes/macrophages could induce the release of soluble factors, e.g. cytokines and chemokines, which play a role in the development of inflammatory responses and in pathogenesis. For example, TNF-α released by Marburg virus-activated monocytes/macrophages increased paraendothelial permeability of human vascular endothelial cells in culture [Feldmann et al., 1996]. It has also been shown recently that TNF-α treatment of endothelial cells inhibits constitu-

TABLE IV. Kinetics of IL-8 Release by HUVEC Infected With Lassa and Mopeia Viruses

	IL-8, ng/ml (mean value ± SD):				
Infection	0	6	12	24	48 hours p. i.
Mock	0.02 ± 0.02	0.92 ± 0.11	1.69 ± 0.26	2.61 ± 0.20	3.54 ± 0.47
Lassa	0.02 ± 0.01	0.41 ± 0.03	0.82 ± 0.18	0.77 ± 0.11	0.67 ± 0.24
Mopeia	0.02 ± 0.08	0.72 ± 0.17	2.69 ± 0.22	3.43 ± 0.26	4.50 ± 0.48

TABLE V. Real-Time PCR Detection of IL-8 mRNA in HUVEC Infected With Lassa and Mopeia Viruses[a]

	Threshold Cycle (C_T):		40/20 hours:	
Infection	IL-8 mRNA	GAPDH mRNA	ΔCT IL-8[b]	ΔIL-8
HUVEC-mock	26.55 ± 0.20	32.42 ± 0.24		
HUVEC-Lassa-20	26.78 ± 0.26	31.65 ± 0.36		
HUVEC-Lassa-40	29.40 ± 0.03	32.95 ± 0.16	(−)1.32	(−)2.5×
HUVEC-Mopeia-20	27.43 ± 0.08	30.64 ± 0.29		
HUVEC-Mopeia-40	29.50 ± 0.49	32.84 ± 0.30	0.13	0.91×

[a]cDNAs were made from RNA extracted from mock or Lassa-, Mopeia-infected cells at 20 and 40 hours p. i. and amplified with IL-8 and GAPDH primers using the GeneAmp 5700 System with SYBR Green I chemistry. Amplification plots were analyzed with 5700 SDS software and expressed as C_T values. The C_T is a PCR cycle at which a statistically significant increase in fluorescence can be detected above the baseline. All PCR reactions had the identical dissociation curves (not shown).
[b]ΔC_T IL-8 values were normalized and subtracted to HUVEC-mock samples (see text for details).

tive FasL synthesis and up-regulates the expression of the adhesion molecule VCAM-1, thereby facilitating cellular infiltration [Sata and Walsh, 1998]. These data and the extensive intravascular apoptosis demonstrated recently in Ebola virus-infected patients [Baize et al., 1999] indicate that the endothelial damage observed in viral HFs can occur by multiple complex mechanisms.

Lassa virus infection of MDM induced no TNF-α transcription or protein release. Even LPS-stimulated MDM did not respond to Lassa virus infection with TNF-α release, as has been described for guinea pig peritoneal macrophages infected with Pichinde virus [Aronson et al., 1995] or for human MDM infected with influenza virus [Nain et al., 1990]. In contrast, Lassa virus infection of LPS-stimulated MDM attenuated TNF-α mRNA expression during the infection. Similar results were observed in human THP-1 monocytic cells infected with hepatitis B virus (HBV) [Oquendo et al., 1997]. Exposure of unstimulated cells to HBV induced neither TNF-α protein secretion nor TNF-α mRNA accumulation. When PMA + LPS-activated THP-1 cells were infected with HBV TNF-α expression was inhibited at the protein release and mRNA levels.

The Lassa-related Mopeia virus induced low levels of TNF-α mRNA in MDM early (12 hours) p. i. (Fig. 1). A "non-specific" reaction of MDM to a high dose of the virus (MOI = 5) could not be excluded. A similar "non-specific" response of human monocytes was observed in cells exposed to a high dose of heat inactivated influenza A virus (the equivalent to MOI of 2). Within 8 hours of exposure to heat-inactivated influenza more than 100 pg/ml of TNF-α could be detected in culture medium [Bussfeld et al., 1998]. At 24 hours p. i., when the highest Mopeia virus yield was detected, TNF-α mRNA expression was not detectable in infected MDM (Table I). In sum, Lassa and Mopeia infections are unlike those of Marburg, influenza, and Pichinde viruses and similar to HBV in their failure to elicit TNF-α expression.

IL-8, the most extensively studied chemokine, plays a pivotal role in inflammation [Ben-Baruch et al., 1995; Ramshaw et al., 1997; Rollins, 1997; Ward et al., 1998]. The published results on the viral inducibility of IL-8 are rather contradictory. Recent publications report that virus infections (measles virus, influenza, respiratory syncytial virus, rhinovirus, rotavirus, cytomegalovirus), induce IL-8 gene expression in cultured cells of the macrophage lineage, in T lymphocytes, and in non-hematopoietic cells (fibroblasts, epithelial and endothelial cells) [van Damme et al., 1989; Becker et al., 1991; Choi et al., 1992; Arnold et al., 1994; Subauste et al., 1995; Johnston et al., 1998]. In vivo increased levels of IL-8 have been found in nasal lavage as a result of acute experimental rhino- and influenza-virus infections. IL-8 expression has been directly associated with inflammation in patients with chronic hepatitis C infection [Grunberg et al., 1997; Murayama et al., 1998; Shimoda et al., 1998]. As a result of respiratory syncytial virus (RSV) infection, IL-8 is found in circulating plasma. A striking correlation between increased levels of IL-8 and severity of disease was found in patients with Dengue HF [Raghupathy et al., 1998]. On the other hand, cell culture experiments with RSV showed that infection of alveolar macrophages slightly increased IL-8 production but that in the presence of LPS, infection induced IL-10 which resulted in an overall suppression of IL-8 production [Panuska et al., 1995]. Bussfeld et al. (1998) found that the expression and release of IL-8 was not inducible in human monocyte cultures infected with influenza A. The discrepancies between cell culture results and *in vivo* measurements of IL-8 may be due to the sensitivity of IL-8 gene

induction to changes in cell adherence or attachment [Kasahara et al., 1991; Shibata et al., 1996].

Human beings afflicted with Lassa fever are usually also infected with other pathogens, some of which may contribute to higher plasma levels of endotoxin (such as LPS). Therefore, it is reasonable to perform infection studies on LPS-activated MDM. Mock-infected MDM released IL-8 in culture medium and were highly inducible for IL-8 expression upon LPS exposure (Table II). Lassa and, to a much lower extent, Mopeia virus infection of MDM suppressed constitutive IL-8 expression (Fig. 3). This suppression required virus replication because gamma-irradiated Lassa virus did not suppress MDM IL-8 release. Lassa virus infection but not Mopeia infection suppressed LPS-induced IL-8 mRNA expression early after infection/stimulation (Figs. 3, 4). Thus, although Lassa suppression of IL-8 in activated MDM was substantial, it is not known whether increased IL-8 suppression could contribute to pathogenesis during concurrent infections of Lassa and endotoxin-producing pathogens.

Lassa virus infection of human endothelial (HUVEC) cultures resulted in significant suppression of IL-8 release as early as 6 hours p. i. (Table IV). Under the same experimental conditions, Mopeia virus infection had a slight stimulatory effect on IL-8 production (Table V). Real-time quantitative PCR data confirmed the ELISA results suggesting that suppression of IL-8 mRNA expression was associated with Lassa but not with Mopeia virus replication in HUVEC.

The opposite effects of Lassa and Mopeia infection on IL-8 expression could be connected with their remarkably different pathogenic potentials in vivo. However, it is difficult to extrapolate these results to animal models or to human beings in the absence of further information. The cyto/chemokine proinflammatory cascade is extremely complex, and at this point we do not know whether IL-8 suppression affects Lassa HF pathogenesis in man. IL-8 activates neutrophils and causes them to migrate to sites of inflammation [Ben-Barush et al., 1995; Rollins, 1997]. It is possible that Lassa suppresses neutrophil activation and thereby evades an early form of host defense. This could contribute to the greater virulence of Lassa over Mopeia, a virus which does not suppress IL-8 in HUVEC. Measurement of IL-8 as well as other proinflammatory cyto/chemokines in plasma of monkeys infected with either virus or in Lassa HF patients at the different stages of the disease will help to clarify the role of these factors in pathogenesis. Based on the our data, we can suggest that the cumulative down-regulation of IL-8 and TNF-α expression could provide a mechanism for the inflammatory and immune response deficiency in severe cases of Lassa HF.

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